REMARKS

Claim 22 is being added. No claims are being cancelled. Claims 1, 10 and 14-15 are being amended. Upon entry of the amendment claims 1-10, 12 and 14-22 will be pending in the application.

The added provisos in claims 1, 10, 14 and 15 are allowable under the instruction of MPEP §2173.05(i) and do not enter new matter. The provisos are also supported by the court decisions that have allowed an applicant to claim a narrowed species within an originally claimed genus. See In re Wertheim, 191 USPQ 90, 97 (CCPA 1976).

New claim 22 is supported by the specification (International Publication No. PCT/US99/28136) at page 18, lines 20-27 which exemplifies some structures falling within cyclic, polycyclic and heterocyclic groups.

The rejection of claims 14-15 under 35 U.S.C. §112, second paragraph.

Claims 14-15 were rejected under 35 U.S.C. §112, second paragraph as being incomplete. The Office communication indicates that claims 14 and 15 recite the presence of a moiety containing "Z" without defining what "Z" can be. Applicant has amended claims 14 and 15 so that "Z" is no longer a possible moiety. Claims 14 and 15 are in condition to overcome this rejection.

The rejection of claims 1, 6, 8-10, 12, 14-17 and 19-21 under 35 U.S.C. §102(b).

Claims 1, 6, 8-10, 12, 14-17 and 19-21 were rejected under 35 U.S.C. §102(b) as having each and every feature and interrelationship anticipated by Lin S. et al; "Novel Analogues of arachidonylethanolamide (anandamide): affinities for the CB1 and CB2 Cannabinoid Receptors and Metabolic Stability"; J. Med. Chem.; vol. 41; 5353; 1998. Applicant is enclosing a copy of the Lin article, which indicates that this article was published on the web on December 12, 1998.

The present 09/600,786 application is the U.S. National Phase of International Application No. PCT/US99/28136. This International Application claims priority from

U.S. Provisional Patent Application No. 60/109,615 filed on November 24, 1998. Thus, the present application has a priority date that is earlier than the publication date of the Lin et al article.

Additionally, U.S. Provisional Patent Application No. 60/109,615 included as part of that application a galley proof for the Lin S. et al publication "Novel Analogues of arachidonylethanolamide (anandamide): affinities for the CB1 and CB2 Cannabinoid Receptors and Metabolic Stability". This galley proof was subsequently published in the J. Med. Chem.; vol. 41; 5353; 1998 that was cited by the Examiner.

The Lin article does not appear to be properly prior art against the present application and applicant respectfully traverses the rejection of claims 1, 6, 8-10, 12, 14-17 and 19-21 over the Lin article.

In summary, Applicants have addressed each of the objections and rejections within the present Office Action. It is believed the application now stands in condition for allowance, and prompt favorable action thereon is respectfully solicited.

The Examiner is invited to telephone Applicant(s)' attorney if it is deemed that a telephone conversation will hasten prosecution of this application.

Respectfully submitted,

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Articles

Novel Analogues of Arachidonylethanolamide (Anandamide): Affinities for the CB1 and CB2 Cannabinoid Receptors and Metabolic Stability

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Several analogues of the endogenous cannabinoid receptor ligand arachidonylethanolamide (anandamide) were synthesized and evaluated in order to study (a) the structural requirements for high-affinity binding to the CB1 and CB2 cannabinoid receptors and (b) their hydrolytic stability toward anandamide amidase. The series reported here was aimed at exploring structure–activity relationships (SAR) primarily with regard to stereoelectronic requirements of ethanolamido headgroup for interaction with the cannabinoid receptor active site. Receptor affinities, reported as K_i values, were obtained by a standard receptor binding assay using [^{3}H]CP-55,940 as the radioligand, while stability toward the amidase was evaluated by comparing the K_i of each analogue in the presence and absence of phenylmethanesulfonyl fluoride (PMSF), a serine protease blocker and inhibitor of anandamide amidase. Introduction of a methyl group in the 1′- and 2′-positions or substitution of the ethanolamido headgroup with a butylamido group gave analogues with vastly improved biochemical stability. This is accomplished in some cases with increased receptor affinity. Conversely, oxazolyl and methyloxazolyl headgroups led to low-affinity analogues. Substitution of the hydroxyl group with electronegative substituents such as fluoro, chloro, allyl, and propargyl groups significantly increased receptor affinity but did not influence the biochemical stability. The 2′-chloro analogue of anandamide was found to have the highest affinity for CB1. Additionally, reversing the positions of the carbonyl and NH in the amido group produces retro-anandamides possessing considerably higher metabolic stability. Replacement of the arachidonyl tail with oleyl or linoleyl results in analogues with low affinities for both receptors. All of the analogues in this study showed high selectivity for the CB1 receptor over the peripheral CB2 receptor. The most potent analogues were tested for their ability to stimulate the binding of [^{35}S]GTPγS to G-proteins and were shown to be potent cannabimimetic agonists. The results are discussed in terms of pharmacophoric features affecting receptor affinity and enzymatic stability.

Introduction

Our understanding of the molecular basis of cannabinoid activity has progressed dramatically over the past decade following the characterization of a saturable and high-affinity binding site in rat brain membranes using the tritiated high-affinity classical cannabinoid ligand [^{3}H]CP-55,940.[1] Concurrently, cannabinoids were shown to induce at least some of their effects by inhibiting adenylate cyclase.[2] These discoveries were followed by the characterization of two cannabinoid receptors: the first (CB1) in mammalian brain[3] and the second (CB2) in the periphery of the spleen.[4] Both of these receptors were cloned and expressed in a variety of cell cultures.[4–6] More recently, an endogenous cannabinoid ligand was isolated from porcine brain, identified as arachidonylethanolamide (anandamide), and shown to bind to the brain cannabinoid receptor with an affinity approximately equal to that of (−)-Δ^9-tetrahydrocannabinol (Δ^9-THC), the active constituent of Cannabis.[7] Furthermore, the biochemical and pharmacological profiles of anandamide are similar to those of a cannabinoid agonist.[8–10] This discovery prompted a plethora of studies addressing various structural, biochemical, and pharmacological issues involving the interactions of anandamide with cannabinoid receptors. Like other cannabimimetic agents, anandamide has been shown to modulate cAMP levels,[8] inhibit N-type calcium currents through a pertussis toxin-sensitive pathway in N18 neuroblastoma cells,[11] and inhibit the electrically evoked twitch response of the mouse vas deferens.[12,13] Anandamide was also shown to produce a series of behavioral responses such as hypothermia, analgesia, catalepsy, and hypoactivity which are characteristic of cannabinoids.[14–18]

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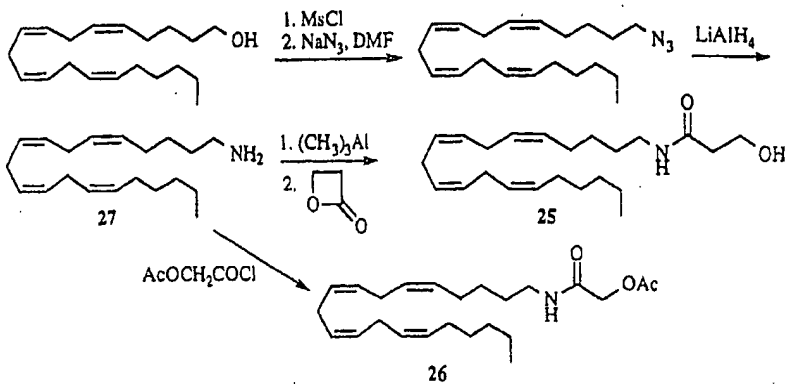
Scheme 1



Anandamide differs pharmacologically from Δ^9-THC in that it has a rapid onset and shorter duration of action and also exhibits somewhat different antinociceptive properties.[16,19] Although the exact physiological role of anandamide is not clearly understood, it is now known that the enzyme anandamide amidase[20-23] present in rat brain microsomes is responsible for its breakdown. This enzymatic breakdown can be prevented, in vitro, by inclusion of PMSF, a nonselective serine protease inhibitor, in the binding assay. Anandamide amidase was shown to be present in regions of rat brain such as the hippocampus where cannabinoid receptor density is the highest.[21] Neuroblastoma cell lines have also been shown to contain anandamide amidase.[21] Two other unsaturated fatty acid ethanolamides have been isolated from porcine brain and shown to be cannabimimetic.[24] It is believed that these compounds behave as lipid mediators in the biochemical and neuronal pathways in the central nervous system.

Structure–activity relationship (SAR) studies of anandamide analogues are just emerging.[25-29] Most of the structural modifications reported to date are in the ethanolamido headgroup. Our initial efforts culminated in the synthesis of a high-affinity and metabolically stable analogue of anandamide, (*R*)-methanandamide.[29] This chiral analogue had 4 times higher affinity than anandamide in the CB1 assay and was found to be relatively stable toward hydrolysis by anandamide amidase. Pinto et al.[26] have reported cannabinoid receptor binding and agonist activity of a number of arachidonyl esters and amides as well as several prostaglandin amides. Arachidonyl esters were found to be much less potent than the corresponding amides.[26] Among the arachidonamides, the propanolamide was shown to exhibit optimal affinity for this series, while *N*-propylarachidonamide which lacks a hydroxyl group at the C-3′ position was shown to have the highest affinity. In another study, Adams et al.[25,27] showed that methylations at the C-2 position resulted in high affinity and some metabolic stability. In the ethanolamido group, replacement of the hydroxy by a fluoro also resulted in a marked increase in affinity, while replacement of the tetraolefinic carbon chain by partially or completely saturated carbon chains resulted in loss of receptor affinity.[25]

Recently we have reported[28] several headgroup analogues of anandamide which show that the structural requirements for the headgroup are rather stringent. In this publication, we describe the synthesis and cannabinoid receptor binding affinity of several novel anandamide analogues. These analogues were designed to further explore stereochemical requirements of the anandamide headgroup for CB1 and CB2 binding and selectivity toward these two cannabimimetic binding sites as well as the effect of headgroup stereochemistry on metabolic stability. Additionally, we explore the role of electron density at the headgroup on receptor affinity and biochemical stability. The role of unsaturation in the fatty acid carbon chain on receptor affinity and selectivity is also explored. Among the analogues reported here, chloroanandamide (**13**) was found to have the highest affinity for CB1 (K_i 5.29 nM). Analogues possessing a methyl group in the 1′- and 2′-positions of the headgroup exhibited improved biochemical stability. However, this modification did not always lead to increased receptor affinity. We also observed that reversal of the carbonyl and NH in the amido group leads to analogues (retro-anandamides) possessing higher metabolic stability.

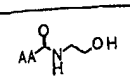
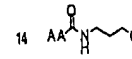
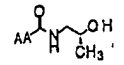
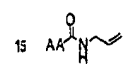
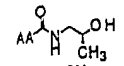
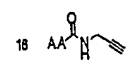
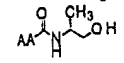
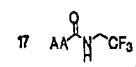
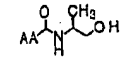
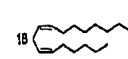
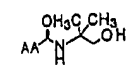
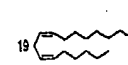
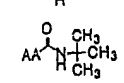
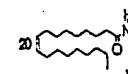
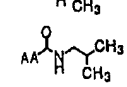
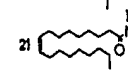
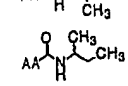
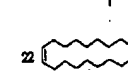
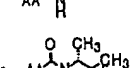
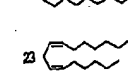
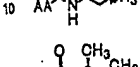
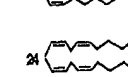
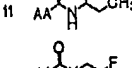
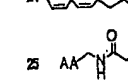
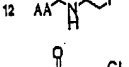
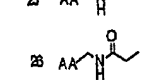
Our overall goal is to develop anandamide analogues possessing high receptor affinity as well as metabolic stability. For this reason, each analogue was subjected to CB1 and CB2 receptor binding assays using rat brain and mouse spleen, respectively, as a source of cannabinoid receptors. The metabolic stability of the anandamide analogues synthesized was assessed by conducting receptor binding assays in the presence and absence of PMSF, a nonselective protease inhibitor which was also shown to inhibit anandamide amidase.[20] Since the mouse spleen preparation does not contain anandamide amidase,[21,30] these assays were performed without PMSF.

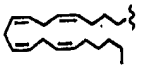
Chemistry

The novel fatty acid amides described here were synthesized by treatment of the appropriate fatty acid chloride, prepared by reaction of the fatty acid in benzene with 2 equiv of oxalyl chloride in the presence of 1 equiv of dimethylformamide at 0 °C, with 10 equiv of an appropriate amino alcohol in tetrahydrofuran under a nitrogen atmosphere.[7,29,31] The oxazoline derivatives **22**–**24** were obtained when the corresponding amides were refluxed with tetrabutylammoniun fluoride in the presence of *p*-toluenesulfonyl fluoride in THF.[32]

Retro-anandamides **25** and **26** were synthesized starting from commercially available arachidonyl alcohol as outlined in Scheme 1. Thus, conversion of the hydroxyl

Table 1. Affinities (K_i) of Anandamide Analogues for Cannabinoid Receptors

		Affinity for the cannabinoid receptors [a] K_i (nM)					Affinity for the cannabinoid receptors [a] K_i (nM)		
		CB1		CB2			CB1		CB2
	Compound [b]	PMSF	No PMSF		Compound [b]	PMSF	No PMSF		
1		61.0* (53.4, 69.7)	5810* (4870, 6790)	1830 (1560, 2380)	14 	25.6 (24.3, 29.1)	387 (358, 422)		193 (184, 203)
2		130* (111, 153)	630* (480, 820)	1760 (1620, 1980)	15 	9.91 (8.79, 11.2)	2990 (2540, 3490)		226 (201, 255)
3		35.6* (30.8, 41.2)	110* (101, 120)	771 (686, 866)	16 	10.8 (9.82, 11.8)	4900 (4250, 5640)		290 (262, 321)
4		17.9* (16.2, 19.7)	28.3* (23.0, 34.9)	868 (758, 997)	17 	38.2 (32.4, 40.4)	2990 (2120, 2680)		718 (504, 854)
5		309* (281, 340)	585* (459, 757)	8220 (7310, 9230)	18 	1.0×10^4 (9.9×10^3, 1.2×10^4)	4.0×10^4 (3.4×10^4, 4.7×10^4)		2.5×10^4 (1.8×10^4, 3.6×10^4)
6		151 (138, 166)	189 (171, 209)	3690 (3310, 4120)	19 	2.1×10^4 (1.9×10^4, 2.3×10^4)	2.1×10^4 (1.4×10^4, 3.0×10^4)		2.1×10^4 (1.7×10^4, 2.6×10^4)
7		51.2 (48.9, 55.9)	105 (98.0, 114)	1140 (1000, 1300)	20 	2.0×10^4 (1.8×10^4, 2.3×10^4)	4.4×10^4 (3.8×10^4, 5.1×10^4)		3.1×10^4 (2.6×10^4, 4.5×10^4)
8		90.2 (82.9, 98.2)	98.1 (89.3, 108)	840 (557, 1270)	21 	1.9×10^4* (1.7×10^4, 2.1×10^4)	3.3×10^4 (1.9×10^4, 5.7×10^4)		2.6×10^4 (2.0×10^4, 3.5×10^4)
9		83.2 (76.3, 90.7)	158 (139, 180)	2200 (1850, 2610)	22 	ND	1.4×10^4 (1.2×10^4, 1.7×10^4)		3.1×10^4 (2.0×10^4, 4.7×10^4)
10		73.6 (69.6, 77.8)	143 (131, 155)	1330 (1090, 1630)	23 	ND	6090 (7050, 9280)		2.0×10^4 (1.6×10^4, 2.5×10^4)
11		297 (267, 309)	379 (354, 405)	3200 (2380, 4320)	24 	ND	679 (581, 793)		6.8×10^5 (7.7×10^5, 6.1×10^7)
12		28.7 (20.4, 34.8)	4640 (3910, 5500)	908 (777, 1060)	25 	115 (106, 124)	134 (121, 149)		3540 (3000, 4040)
13		5.29 (4.76, 5.88)	3400 (2940, 3930)	195 (174, 219)	26 	421 (360, 493)	419 (358, 491)		>10000

[a] The 95% confidence limits are in parentheses. Affinity of anandamide and its analogues for the cannabinoid receptor was determined using rat brain membranes and 0.8 nM [^{3}H]CP-55,940 as the radioligand essentially as previously described.[29,33] Data were analyzed using nonlinear regression analysis. K_i values were obtained from a minimum of two independent experiments run in duplicate and are expressed as the mean ± standard error. *The data are different from that in the earlier published paper[29] due to the new Top-Count procedure. ND, not determined. [b] AA= 

group to the mesylate followed by displacement with sodium azide gave the intermediate azide which was reduced to arachidonylamine using lithium aluminum hydride. Reaction of this amine with trimethylaluminum followed by β-propiolactone gave retro-anandamide 25, while reaction with acetoxyacetyl chloride gave analogue 26.

Results and Discussion

The objective of this study was to design and synthesize anandamide analogues with high receptor affinity, receptor selectivity, and metabolic stability toward anandamide amidase, the enzyme responsible for the breakdown of anandamide into arachidonic acid and ethanolamine. In an earlier publication,[29] we have reported the first metabolically stable, chiral anandamide analogue (*R*)-methanandamide (4) which was shown to have remarkable enzymatic stability and about 4-fold higher CB1 receptor affinity than anandamide.

CB1 and CB2 Cannabinoid Receptor Affinities of Anandamide Analogues. Prior to assaying the newly synthesized analogues for CB1 affinity, rat forebrain synaptosomal membranes were treated with 150 μM PMSF as described elsewhere.[29] The treatment was based on recent accounts providing evidence for amidase activity in rat brain membrane preparations that results in the hydrolysis of anandamide and is inhibited by PMSF.[20,21] The analogues were also tested for CB1 receptor affinity without PMSF. CB2 receptor affinity was measured using mouse spleen membranes where anandamide amidase activity is absent[21,30] and does not require pretreatment with PMSF. Details of the assay have been described earlier.[33] A minimum of three independent experiments conducted in duplicate were used to define the IC_{50} for each compound. IC_{50} values obtained by nonlinear least-squares fitting of the data were converted to K_i values using the Cheng–Prusoff equation[34] with a K_d for [^{3}H]CP-55,940 of 0.85 nM. The data are presented in Table 1 which includes, for the

purpose of comparison, analogues 2–5 reported earlier by our laboratory.[29] Introduction of a second methyl group at C-1′ to afford the *gem*-dimethyl analogue 6 resulted in considerable loss of CB1 receptor affinity. However, the *tert*-butyl analogue 7 showed better affinity for CB1 than 6 and anandamide indicating that a sterically bulky hydrophobic group is tolerated in the C-1′ position of anandamide. Adams and co-workers[25,27] have reported C-2 (α to the carbonyl group) alkyl analogues of anandamide and have also shown that analogues containing a methyl or a *gem*-dimethyl group at C-2 exhibit better CB1 receptor affinity and metabolic stability when compared to anandamide. However, the corresponding C-2 isopropyl analogue was found to have very weak affinity for CB1. In an earlier study, Pinto et al.[26] showed that the hydroxyl group of anandamide is not a requirement for binding at the active site. Thus, *N*-propylarachidonamide was found to have an affinity 3 times higher than that of anandamide. We have made a similar observation in the case of the *tert*-butylamido analogue 7 which had a higher affinity (K_i 51.2 nM) than the corresponding monohydroxy analogue 6 (K_i 151 nM) and anandamide. However, our results show that this finding cannot be generalized. For example, in the case of (*R*)-methanandamide (4) removal of the hydroxyl group results in analogue 10 with about 4-fold lower affinity. Similarly, (*S*)-methanandamide (5) and its alkyl analogue 11 have comparable CB1 receptor affinities.

The above results suggest the existence of a hydrophobic subsite for the anandamide headgroup in both CB1 and CB2, capable of accommodating relatively bulky substituents. The results also indicate that the bulky arachidonalkylamides may bind differently than their hydroxyalkyl counterparts including anandamide.

We have also explored the effect of electronegative headgroup substituents on cannabinoid receptor affinity. Introduction of unsaturation, e.g., a double or triple bond, in the headgroup gave analogues with severalfold higher affinity than that of anandamide. Thus, *N*-allylarachidonamide (15) (K_i 9.91 nM) and *N*-propargylarachidonamide (16) (K_i 10.8 nM) showed approximately 6-fold higher affinity for CB1 than anandamide (K_i 61.0 nM). We also explored the effect of halogen substitution in the headgroup and found that replacement of the hydroxyl group by fluorine (analogue 12) resulted in an increase in affinity. Its K_i value has been previously reported to be 8.60 nM.[25] In our assay, we obtained a K_i value of 26.7 nM in the presence of PMSF. This may be due to variations of assay conditions between the two laboratories. The 2′,2′,2′-trifluoroethyl analogue 17 was found to be about twice as potent as anandamide. However, the most potent of the haloanandamides was 2′-chloroanandamide (13) which was found to have the highest affinity among the analogues reported here. Its K_i value was approximately 12- and 3-fold higher than that of anandamide and (*R*)-methanandamide, respectively. One-carbon homologation of 13 to the *N*-3-chloropropyl analogue 14 resulted in a 5-fold decrease in affinity for CB1. However, this compound still demonstrated a 2-fold higher affinity for CB1 than anandamide and the highest affinity for CB2 (K_i 193 nM) relative to CB1.

The novel retro-analogues (25, 26) of anandamide, in which the positions of the carbonyl and NH in the amido group have been reversed, showed somewhat weaker CB1 affinity compared to anandamide with 25 exhibiting a K_i for CB1 slightly more than 2-fold that of anandamide, while the acetyl analogue 26 had an affinity 7-fold lower than that of anandamide. Also, both retro-analogues had very low affinity for CB2. In stark contrast to anandamide, however, both 25 and 26 exhibited essentially no change in their K_i values for CB1 in the absence of PMSF indicating that they were not susceptible to hydrolysis by anandamide amidase.

We tested two other C-18 fatty acid amides for cannabinoid receptor affinity in order to explore the role of the tetraolefinic carbon chain of anandamide. Ethanolamides of the monoolefinic oleic acid (20) and the diolefinic linoleic acid (18) were found to have very weak affinity for both CB1 and CB2. The corresponding (*R*)-methanandamide analogues 21 and 19 of these two fatty acids were also found to have weak affinity for the two receptors. This lack of affinity could be due to conformational differences in the fatty acid component of the analogues. In this regard, it has been demonstrated by computational studies[35] that the minimum-energy conformation of anandamide is a loop (hairpin) conformation which may also be the active conformation. Arguably, the reduced receptor affinities of these mono- and disolefinic amides can be attributed to their preferred conformations which are expected to be different than the conformation of anandamide.

The oxazoline derivatives 22–24 were byproducts obtained during the fluorination reaction. These analogues had only weak affinities for CB1 and practically no affinity for CB2. These results suggest that the presence of a free NH is important for receptor binding possibly through hydrogen bonding. We have reported[28] similar observations in a recent article in which anandamide analogues with a tertiary nitrogen were found to be inactive except for one with bis(hydroxyethyl) substitution perhaps capable of engaging in H-bonding with the receptor.

In general, anandamide analogues reported in this article exhibited 10–40-fold selectivity for the CB1 receptor with the exception of 14 which had only an 8-fold selectivity for CB1 over CB2. So far no CB2-selective anandamide analogue has been reported. The remarkable preference of anandamides for the CB1 receptor makes them an important class of CB1-selective cannabimimetic ligands.

While this manuscript was in preparation, Sheskin et al. published[36] an article describing structural requirements of anandamide analogues for binding to CB1. They reported K_i values for analogues 7, 10, and 11 of 138.6, 239.9, and 377.2 nM, respectively. These values are generally higher than those reported in this article. The discrepancy could be attributed to the use of different assay conditions and radioligands by the above authors.

Biochemical Stability of Anandamide Analogues. One of the objectives of the present study was to develop anandamide analogues with biochemical stability toward anandamide amidase, the enzyme responsible for the breakdown of anandamide-like fatty acid amides.[20] The biochemical stability of analogues described here was assessed by comparing their affinities for the CB1

receptor in the presence and absence of phenylmethanesulfonyl fluoride (PMSF), a common protease inhibitor. It is well-known that hydrolysis of an ester or amide bond can be inhibited by the introduction of sterically bulky groups in close proximity to the bond. Using this rationale, in an earlier publication we have reported[29] four methyl analogues of anandamide, of which (*R*)-methanandamide was found to have excellent biochemical stability as well as CB1 receptor affinity 4-fold higher than that of anandamide. We now report several new analogues containing alkyl groups in the ethanolamido headgroup. As can be seen from the data in Table 1, the C-2′ *gem*-dimethyl analogue **6** showed very good biochemical stability toward the amidase since the presence of PMSF had no effect on its affinity for CB1. However, the presence of two methyl groups resulted in reduced affinity for CB1, approximately 8-fold lower than that of (*R*)-methanandamide. Conversely, the *tert*-butyl analogue **7** had an almost equal biochemical stability to that of **6** but also had a 3-fold higher affinity for CB1. As for the enantiomeric pair of isobutyl analogues **10** and **11**, the *R*-enantiomer showed 3-fold higher metabolic stability than the *S*-enantiomer. Also noteworthy is the fact that **10** exhibits about 4 times higher affinity for CB1 than its enantiomer **11**. This is reminiscent of our earlier observation[29] that (*R*)-methanandamide is superior to (*S*)-methanandamide with regard to both CB1 affinity and enzymatic stability. An equally interesting observation is that *N*-isobutylarachidonamide (**8**) exhibits a high degree of biochemical stability, even higher than the respective β-methylethanolamide analogues **2** and **3**. This difference in substrate selectivity toward the enzyme could be attributed to stereochemical differences in the manner in which the *N*-alkyl (**8**) and *N*-hydroxyalkyl (**2**, **3**) analogues interact with the enzyme's binding site.

As mentioned earlier, the retro-anandamides **25** and **26** showed somewhat weaker CB1 affinity than anandamide. However, in stark contrast to anandamide, these compounds exhibit excellent stability toward enzymatic hydrolysis.

As a general rule we have observed that increased electronegativity at the headgroup is accompanied with decreased biochemical stability. Thus the fluoro (**12**), chloro (**13**), and trifluoroethyl (**17**) headgroup analogues exhibited reduced biochemical stability. Similarly, the electronegative allylamido (**15**) and propargylamido (**16**) substituents led to analogues with apparently lower metabolic stability than the respective alkyl analogues and anandamide itself. Conversely, when the electronegative substituent was separated by three carbon atoms from the amide group, as in the case of 3′-chloropropylanandamide (**14**), metabolic stability was partially restored. The poor biochemical stability of the haloethyl and two-carbon unsaturated headgroup analogues of anandamide could be due to the effects of these electronegative substituents on the carbonyl group. Indeed, the carbonyl carbon in the above analogues is more electrophilic in nature compared to the corresponding *N*-alkyl analogues or anandamide and hence more susceptible to a nucleophilic attack by the amidase. Such an inference is corroborated by the fact that the 3-chloropropyl analogue **14** is hydrolytically much more stable as compared to the 2-chloroethyl analogue **13**.

Table 2. EC_{50} and E_{max} Values of Anandamide Analogues for Stimulating [^{35}S]GTPγS Binding in Rat Microsomal Membranes[a]

analogue	EC_{50} (μM)	log EC_{50} (μM)	E_{max} (%)
13	0.42 ± 1.7	−0.4 ± 0.2	162 ± 5.8
16	0.10 ± 6.2	−1.7 ± 0.7	143 ± 2.5
4	0.33 ± 1.8	−0.5 ± 0.3	148 ± 3.9
WIN 55,212-2	0.18 ± 0.09	−0.73 ± 0.23	150 ± 8.9
CP-55,940	0.10 ± 0.05	−1.7 ± 0.53	145 ± 12.0

[a] [^{35}S]GTPγS binding assay was conducted using eight concentrations of each analogue being tested and two experiments run with four duplicates of each point.

[^{35}S]GTPγS Binding Assay of Anandamide Analogues. Cannabinoid receptors belong to the G-protein-coupled receptor (GPCR) superfamily and are known to inhibit the enzyme adenylate cyclase. It is also known that cannabinoid agonists stimulate the binding of [^{35}S]GTPγS to G-proteins in the presence of excess GDP.[38,39] Three representative anandamide analogues possessing high receptor affinity described in this article were subjected to the [^{35}S]GTPγS binding assay in order to assess their functional potency as agonists or antagonists. The data for analogues **4** ((*R*)-methanandamide), **13** (chloro) and **16** (propargyl) are shown in Table 2. As can be seen from the data, all three analogues had a dose-dependent stimulation on [^{35}S]GTPγS binding thus behaving as CB1 agonists in agreement with results from other anandamide analogues.[29,38] The chloro analogue **13** showed the highest stimulation (E_{max} = 162%) while the propargyl analogue **16** was found to have the highest potency (EC_{50} = 0.10 μM) among the three analogues tested. The data shown here indicate that all three compounds are potent agonists with potencies comparable to those of CP-55,940 and WIN 55,212−2 which were also assayed under identical conditions.

Conclusions

In this article, we have shown that structural modification of the ethanolamido headgroup of anandamide affects both cannabinoid receptor affinity and the biochemical stability. Introduction of alkyl group(s) in the headgroup imparts biochemical stability toward the action of anandamide amidase and, in some cases, leads to a concomitant increase in receptor affinity. Also, introduction of electronegative groups such as halogens or unsaturated three-carbon substituents in the headgroup results in marked increase in receptor affinity. However, such analogues exhibit reduced biochemical stability. Interestingly, switching the positions of the carbonyl and the NH groups results in metabolically stable anandamide analogues, although receptor binding affinity is somewhat diminished when compared to that of anandamide. This is a significant step toward the development of metabolically stable anandamides. In general, anandamide analogues show 10−40-fold selectivity toward the CB1 cannabinoid receptor as compared to CB2 receptor.

The above data provide insights on the interactions between the anandamide headgroup and the CB1 receptor sites as well as on the selectivity of anandamides for CB1 versus CB2. Furthemore, semiquanti-

tative data are included regarding the biochemical stability of anandamides with respect to anandamide amidase activity. Such information can be of value for the design of novel metabolically stable analogues possessing higher affinity for the CB1 cannabinoid receptor.

Experimental Section

General. ^{1}H NMR spectra were recorded on either a 200- or 270-MHz spectrometer using TMS as the internal standard. All chemical shifts are reported in ppm. Elemental analyses were obtained for the newly synthesized analogues and are within ± 0.4% of theoretical values. Specific rotations were determined using a Perkin-Elmer 241 polarimeter within a 1.00-dm cell. Optically pure amino alcohols and amines were obtained from Aldrich Chemical Co. (Milwaukee, WI). Arachidonic acid was purchased from Nu-Chek-Prep, Inc., Elysian, MN. Melting points were determined on a Thomas-Hoover melting point apparatus.

Rat brains were purchased from Pelfreeze Rogers, AR. [^{35}S]-GTPγS was purchased from New England Nuclear Corp., Boston, MA, while GDP and GTPγS were purchased from Sigma Chemical Co.

***N*-(1,1-Dimethyl-2-hydroxyethyl)arachidonamide (6).** To a magnetically stirred solution of 200 mg (0.66 mmol) of arachidonic acid and 48.0 mg (0.66 mmol) of DMF in 5 mL of benzene in a 25-mL three-neck flask at 0 °C was added 168 mg (1.32 mmol) of oxalyl chloride in a dropwise manner. The reaction mixture was stirred further at 0 °C for an additional hour. Then 5 mL of THF was added followed by a solution of 588 mg (6.60 mmol) of 2-amino-2-methyl-1-propanol in 5 mL of THF. Stirring was continued further for 15 min. The reaction mixture was diluted with 30 mL of CH_2Cl_2 and washed successively with 30 mL of 10% of aqueous HCl, 30 mL of 10% aqueous NaOH, and water. The organic phase was separated, dried ($MgSO_4$), and evaporated in vacuo to give a yellowish oily residue. Chromatography on silica gel (eluted first with CH_2Cl_2 and then with 1% MeOH−CH_2Cl_2) afforded 200 mg (0.53 mmol, 80.0% yield) of **6** as a light-green colored liquid: R_f (5% MeOH−CH_2Cl_2) 0.40; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.36−5.47 (m, 8H), 3.58 (s, 2H), 2.78−2.83 (m, 6H), 2.00−2.21 (m, 6H), 1.66−1.77 (m, 2H), 1.29 (s, 6H), 1.17−1.35 (m, 6H), 0.89 (t, J = 5.80 Hz, 3H). Anal. ($C_{24}H_{41}NO_2$) C, H, N.

***N*-(1,1-Dimethylethyl)arachidonamide (7).** *N*-(1,1-Dimethylethyl)arachidonamide (**7**) was prepared from 200 mg (0.66 mmol) of arachidonic acid and 498 mg (6.60 mmol) of *tert*-butylamine in benzene following the procedure described for compound **6** to give 220 mg (0.61 mmol, 93.4% yield) of **7** as a colorless oil: R_f (5% MeOH−CH_2Cl_2) 0.25; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.30−5.38 (m, 8H), 2.78−2.89 (m, 6H), 2.06−2.13 (m, 6H), 1.69 (quintet, 2H), 1.30−1.34 (m, 15H), 0.89 (t, J = 6.50 Hz, 3H). Anal. ($C_{24}H_{41}NO$) C, H, N.

***N*-(2-Methylpropyl)arachidonamide (8).** *N*-(2-Methylpropyl)arachidonamide (**8**) was prepared from 200 mg (0.66 mmol) of arachidonic acid and 498 mg (6.60 mmol) of isobutylamine in benzene following the procedure described for compound **6** to give 210 mg (0.58 mmol, 89.2% yield) of **8** as a colorless liquid: R_f (5% MeOH−CH_2Cl_2) 0.30; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.23−5.49 (m, 8H), 3.05−3.11 (m, 2H), 2.81−2.91 (m, 6H), 2.00−2.22 (m, 6H), 1.76 (m, 3H), 1.30 (m, 6H), 0.91 (d, J = 6.70 Hz, 9H). Anal. ($C_{24}H_{41}NO$) C, H, N.

(±)-*N*-(1-Methylpropyl)arachidonamide (9). (±)-*N*-(1-Methylpropyl)arachidonamide (**9**) was prepared from 200 mg (0.66 mmol) of arachidonic acid and 498 mg (6.60 mmol) of (*R*)-*sec*-butylamine in benzene following the procedure described for compound **6** to give 220 mg (0.61 mmol, 93.4% yield) of **9** as a colorless liquid: R_f (CH_2Cl_2) 0.20; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.28−5.47 (m, 8H), 3.84−3.99 (m, 1H), 2.78−2.84 (m, 6H), 2.00−2.30 (m, 6H), 1.71 (quintet, 2H), 1.30−1.52 (m, 8H), 1.11 (d, J = 6.58 Hz, 3H), 0.90 (t, J = 7.38 Hz, 6H). Anal. ($C_{24}H_{41}NO$) C, H, N.

(*R*)-*N*-(1-Methylpropyl)arachidonamide (10). (*R*)-*N*-(1-Methylpropyl)arachidonamide (**10**) was prepared from 200 mg (0.66 mmol) of arachidonic acid and 498 mg (6.60 mmol) of (*R*)-*sec*-butylamine in benzene following the procedure described for compound **6** to give 230 mg (0.64 mmol, 97.7% yield) of **10** as a colorless liquid: R_f (CH_2Cl_2) 0.20; $[\alpha]^{25}_D$ −6.5° (c = 1, CH_2Cl_2); ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.28−5.47 (m, 8H), 3.84−3.99 (m, 1H), 2.78−2.84 (m, 6H), 2.00−2.30 (m, 6H), 1.71 (quintet, 2H), 1.30−1.52 (m, 8H), 1.11 (d, J = 6.58 Hz, 3H), 0.90 (t, J = 7.38 Hz, 6H). Anal. ($C_{24}H_{41}NO$) C, H, N.

(*S*)-*N*-(1-Methylpropyl)arachidonamide (11). (*S*)-*N*-(1-Methylpropyl)arachidonamide (**11**) was prepared from 200 mg (0.66 mmol) of arachidonic acid and 498 mg (6.60 mmol) of (*S*)-*sec*-butylamine in benzene following the procedure described for compound **6** to give 210 mg (0.58 mmol, 89.2% yield) of **11** as a colorless liquid: R_f (CH_2Cl_2) 0.20; $[\alpha]^{25}_D$ +6.5° (c = 1, CH_2Cl_2); ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.28−5.47 (m, 8H), 3.84−3.99 (m, 1H), 2.78−2.84 (m, 6H), 2.00−2.30 (m, 6H), 1.71 (quintet, 2H), 1.30−1.52 (m, 8H), 1.11 (d, J = 6.58 Hz, 3H), 0.90 (t, J = 7.38 Hz, 6H). Anal. ($C_{24}H_{41}NO$) C, H, N.

***N*-(2-Fluoroethyl)arachidonamide (12).** *N*-(2-Fluoroethyl)arachidonamide (**12**) was prepared from 100 mg (0.33 mmol) of arachidonic acid in benzene and 327 mg (3.28 mmol) of fluoroethylamine hydrochloride, which was previously dissolved in 2 mL of 1 equiv of 1 M NaOH, following the procedure for compound **6** to give 86.0 mg (0.25 mmol, 75.0% yield) of **12** as a liquid: R_f (5% MeOH−CH_2Cl_2) 0.85; ^{1}H NMR (200 MHz, $CDCl_3$) 5.30−5.44 (m, 8H), 4.50 (dt, J = 47.4 Hz, 4.72 Hz, 2H), 3.54 (dt, J = 28.6 Hz, 5.08 Hz, 2H), 2.79−2.87 (m, 6H), 2.28 (t, J = 7.60 Hz, 2H), 2.01−2.14 (m, 4H), 1.65−1.80 (m, 2H), 1.30 (br s, 6H), 0.89 (t, J = 6.89 Hz, 3H). Anal. ($C_{22}H_{36}FNO$) C, H, N.

***N*-(2-Chloroethyl)arachidonamide (13).** A solution of arachidonic acid (50 mg, 0.165 mmol) and 0.03 mL of anhydrous DMF in 1 mL of dry dichloromethane was cooled in an ice bath under argon and 0.17 mL of a 2 M solution of oxalyl chloride (0.34 mmol) in dichloromethane was added dropwise. Reaction mixture was stirred further at ice bath temperature for 1 h. A solution of 65 mg (0.50 mmol, 3 equiv) of 2-chloroethylamine hydrochloride in 0.5 mL of dry pyridine was added, the cooling bath was removed, and the solution was stirred at room temperature for 30 min. The mixture was transferred to a separatory funnel, washed with 10% aqueous hydrochloric acid and water, and dried ($MgSO_4$). After rotary evaporation of solvents, the residue was chromatographed on silica gel (60% ethyl ether−petroleum ether) to afford 54 mg (90%) of the pure title compound as an oil: R_f (70% ethyl ether-petroleum ether) 0.35; ^{1}H NMR (270 MHz, $CDCl_3$) 5.80 (br s, 1H), 5.36 (m, 8H), 3.61 (m, 4H), 2.80 (m, 6H), 2.21 (t, J = 7.89 Hz, 2H), 2.10 (m, 4H), 1.72 (m, 2H), 1.29 (m, 6H), 0.88 (t, J = 6.79 Hz, 3H). Anal. ($C_{22}H_{36}ClNO$) C, H, N.

***N*-(3-Chloropropyl)arachidonamide (14).** The title amide was prepared from 50 mg (0.165 mmol) of arachidonic acid as described under **13**: yield 55 mg (88%); R_f (70% ethyl ether−petroleum ether) 0.35; ^{1}H NMR (270 MHz, $CDCl_3$) 5.60 (br s, 1H), 5.36 (m, 8H), 3.57 (t, J = 6.40 Hz, 2H), 3.40 (q, J = 6.45 Hz, 2H), 2.80 (m, 6H), 2.18 (t, J = 7.91 Hz, 2H), 2.01 (m, 6H), 1.70 (m, 2H), 1.29 (m, 6H), 0.88 (t, J = 6.89 Hz, 3H). Anal. ($C_{23}H_{38}ClNO$) C, H, N.

***N*-Allylarachidonamide (15).** Arachidonic acid chloride was prepared from 50 mg (0.165 mmol) of arachidonic acid as described under **13** and treated with 0.061 mL (0.83 mmol, 5 equiv) of allylamine. Similar workup followed by column chromatographic purification gave 48 mg (85%) of the pure title compound as an oil: R_f (40% ethyl ether−petroleum ether) 0.18; ^{1}H NMR (270 MHz, $CDCl_3$) 5.83 (m; 1H), 5.36 (m, 9H), 5.14 (m, 2H), 3.87 (m, 2H), 2.80 (m, 6H), 2.19 (t, J = 7.93 Hz, 2H), 2.10 (m, 4H), 1.72 (m, 2H), 1.29 (m, 6H), 0.88 (t, J = 6.74 Hz, 3H). Anal. ($C_{23}H_{37}NO$) C, H, N.

***N*-Propargylarachidonamide (16).** Arachidonic acid chloride was prepared from 50 mg (0.165 mmol) of arachidonic acid as described above for **13** and treated with 0.057 mL (0.83 mmol, 5 equiv) of propargylamine. Similar workup followed by column chromatographic purification gave 47.8 mg (85%) of the pure title compound as an oil: R_f (70% ethyl ether−petroleum ether) 0.30; ^{1}H NMR (270 MHz, $CDCl_3$) 5.57 (br s,

1H), 5.36 (m, 8H), 4.04 (m, 2H), 2.80 (m, 6H), 2.22–2.00 (m, 7H), 2.01 (m, 6H), 1.72 (m, 2H), 1.29 (m, 6H), 0.88 (t, J = 6.73 Hz, 3H). Anal. ($C_{23}H_{35}NO$) C, H, N.

***N*-(2,2,2-Trifluoroethyl)arachidonamide (17).** Arachidonic acid chloride was prepared from 50 mg (0.165 mmol) of arachidonic acid as described under **13** and reacted with a solution of 111.8 mg (0.825 mmol, 5 equiv) of 2,2,2-trifluoroethylamine hydrochloride in 0.5 mL of pyridine. Reaction mixture was stirred at room temperature for 30 min and worked up in a similar manner to give 47.4 mg (75%) of the title amide: R_f (35% ethyl ether–petroleum ether) 0.20; ^{1}H NMR (270 MHz, $CDCl_3$) 5.60 (br s, 1H), 5.36 (m, 8H), 3.91 (m, 2H), 2.80 (m, 6H), 2.24 (t, J = 7.39 Hz, 2H), 2.08 (m, 6H), 2.01 (m, 6H), 1.74 (m, 2H), 1.29 (m, 6H), 0.88 (t, J = 6.56 Hz, 3H). Anal. ($C_{22}H_{34}F_3NO$) C, H, N.

***N*-(2-Hydroxyethyl)linoleamide (18).** The title amide was prepared from 3.00 g (11.3 mmol) of linoleic acid and 6.90 g (113 mmol) of aminoethanol in benzene following the procedure described for compound **6** to give 2.79 g (8.63 mmol, 76.6% yield) of a white solid: mp 38–39.5 °C; R_f (5% MeOH–CH_2Cl_2) 0.24; ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.00 (br s, 1H), 5.31–5.39 (m, 4H), 3.73 (t, J = 4.68 Hz, 2H), 3.43 (td, J = 5.01 Hz, 5.09 Hz, 2H), 2.77 (t, J = 5.45 Hz, 2H), 2.21 (t, J = 7.54 Hz, 2H), 2.03 (m, 4H), 1.67 (m, 2H), 1.31 (br s, 14H), 0.89 (t, J = 6.30 Hz, 3H). Anal. ($C_{20}H_{37}NO_2$) C, H, N.

***(R)*-*N*-(1-Methyl-2-hydroxyethyl)linoleamide (19).** This amide was prepared from 1.00 g (3.76 mmol) of linoleic acid and 1.41 g (18.8 mmol) of (*R*)-(−)-2-amino-1-propanol in benzene following the procedure described for compound **6** to give 1.07 g (3.17 mmol, 84.5% yield) of **19** as a colorless liquid: R_f (5% MeOH–CH_2Cl_2) 0.21; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.90 (br s, 1 H), 5.22–5.45 (m, 4H), 3.99–4.07 (m, 1H), 3.47–3.66 (m, 3H), 2.77 (t, J = 5.58 Hz, 2H), 2.18 (t, J = 7.50 Hz, 2H), 2.00–2.06 (m, 4H), 1.58–1.65 (m, 2H), 1.30 (br s, 14H), 0.89 (t, J = 6.07 Hz, 3H). Anal. ($C_{21}H_{39}NO_2$) C, H, N.

***N*-(2-Hydroxyethyl)oleamide (20).** Reaction of 3.00 g (10.6 mmol) of oleic acid and 6.50 g (106 mmol) of aminoethanol in benzene following the procedure described for compound **6** gave 2.88 g (8.86 mmol, 83.6% yield) of **20** as a white solid: mp 60–61 °C; R_f (5% MeOH–CH_2Cl_2) 0.28; ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.34 (br s, 1H), 5.34 (m, 2H), 3.70 (t, J = 4.54 Hz, 2H), 3.56 (br s, 1H), 3.41 (m, 2H), 2.20 (t, J = 7.20 Hz, 2H), 2.00 (m, 4H), 1.69 (m, 2H), 1.30 (br s, 20H), 0.88 (t, J = 6.00 Hz, 3H). Anal. ($C_{20}H_{39}NO_2$) C, H, N.

***(R)*-*N*-(1-Methyl-2-hydroxyethyl)oleamide (21).** The title compound was prepared from 1.00 g (3.54 mmol) of oleic acid and 1.33 g (17.5 mmol) of (*R*)-(−)-2-amino-1-propanol in benzene following the procedure described for compound **6** to give 860 mg (2.53 mmol, 71.6% yield) of a white solid: mp 43–44 °C; R_f (5% MeOH–CH_2Cl_2) 0.23; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.83 (br s, 1H), 5.22–5.45 (m, 4H), 3.99–4.07 (m, 1H), 3.54–3.67 (m, 2H), 3.37 (br s, 1H), 2.77 (t, J = 5.58 Hz, 2H), 2.18 (t, J = 6.90 Hz, 2H), 2.00–2.06 (m, 4H), 1.58–1.65 (m, 2H), 1.30 (br s, 14H), 0.88 (t, J = 6.04 Hz, 3H). Anal. ($C_{21}H_{41}NO_2$) C, H, N.

2-(1-Oleyl)oxazoline (22). To a magnetically stirred solution of 604 mg (2.31 mmol) of tetrabutylammonium fluoride in 5 mL of THF was added a mixture of 268 mg (1.54 mmol) of *p*-toluenesulfonyl fluoride and 250 mg (0.77 mmol) of *N*-(2-hydroxyethyl)oleylamide (**20**) in 5 mL of THF at room temperature. After stirring at reflux for 2 h, the reaction mixture was filtered through a pad of Celite. The filtrate was collected and evaporated in vacuo, and the residue was chromatographed on silica gel (10% EtOAc–petroleum ether) to give 114 mg (0.37 mmol, 48.0% yield) of **22** as a colorless liquid: R_f (50% EtOAc–petroleum ether) 0.12; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.32–5.37 (m, 2H), 4.22 (t, J = 9.51 Hz, 2H), 3.82 (t, J = 9.34 Hz, 2H), 2.27 (t, J = 7.52 Hz, 2H), 2.01 (d, J = 5.27 Hz, 4H), 1.59–1.66 (m, 2H), 1.31 (br s, 20H), 0.89 (t, J = 6.24 Hz, 3H). Anal. ($C_{20}H_{37}NO$) C, H, N.

2-(1-Linoleyl)oxazoline (23). This compound was prepared from 250 mg (0.77 mmol) of *N*-(2-hydroxyethyl)linoleamide (**18**), 268 mg (1.54 mmol) of *p*-toluenesulfonyl fluoride, and 604 mg (2.31 mmol) of tetrabutylammonium fluoride in 5 mL of THF following the procedure for compound **22** to give 127 mg (0.54 mmol, 54.5% yield) of **23** as a colorless liquid: R_f (50% EtOAc–petroleum ether) 0.12; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.31–5.39 (m, 4H), 4.22 (t, J = 9.38 Hz, 1H), 3.82 (t, J = 9.41 Hz, 2H), 2.77 (t, J = 5.60 Hz, 2H), 2.27 (t, J = 7.52 Hz, 2H), 2.00–2.06 (m, 4H), 1.62 (m, 2H), 1.30 (br s, 14H), 0.89 (t, J = 6.48 Hz, 3H). Anal. ($C_{20}H_{35}NO$) C, H, N.

2-(1-Arachidonyl)-5(*R*)-methyloxazoline (24). The title compound was prepared from 260 mg (0.72 mmol) of (*R*)-methanandamide (**4**), 268 mg (1.54 mmol) of *p*-toluenesulfonyl fluoride, and 604 mg (2.31 mmol) of tetrabutylammonium fluoride in 5 mL of THF following the procedure for compound **22** to give 124 mg (0.36 mmol, 50.1% yield) of **24** as a colorless liquid: R_f (50% EtOAc–petroleum ether) 0.34; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.23–5.46 (m, 8H), 4.31 (t, J = 8.50 Hz, 1H), 4.06 (m, 1H), 3.74 (t, J = 7.45 Hz, 1H), 2.74–2.84 (m, 6H), 2.28 (t, J = 7.63 Hz, 2H), 2.01–2.19 (m, 4H), 1.63–1.78 (m, 2H), 1.30 (br s, 6H), 1.24 (d, J = 6.34 Hz, 3H), 0.89 (t, J = 6.24 Hz, 3H). Anal. ($C_{23}H_{37}NO$) C, H, N.

Arachidonylamine (27). To a magnetically stirred solution of 50 mg (0.17 mmol) of arachidonyl alcohol in 1 mL of pyridine was added 29.2 mg (0.255 mmol) of mesyl chloride at 0 °C. After stirring for 5 h, the reaction mixture was poured into 2 mL of cold water and extracted with diethyl ether (2 × 4 mL). The combined ether extracts were washed with 1 N sulfuric acid and saturated sodium bicarbonate solution and evaporated in vacuo. The crude mesylate was dissolved in 2 mL of anhydrous DMF, and then a solution of 6.5 mg (0.85 mmol) of sodium azide in 4 mL of anhydrous DMF was added at room temperature. The reaction mixture was heated at 90 °C for 24 h behind a safety shield. The mixture was cooled to room temperature, inorganic material was filtered off, and the filtrate was poured into 1 mL of cold water. Extraction with diethyl ether (2 × 6 mL), drying ($MgSO_4$), and evaporation gave an oily residue which was chromatographed on silica gel (petroleum ether) to afford 39 mg (73%) of arachidonyl azide as a colorless oil: ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.38 (m, 8H), 3.27 (t, J = 6 Hz, 2H), 2.81 (m, 6H), 2.11–2.01 (m, 4H), 1.62 (m, 2H), 1.48–1.25 (m, 6H), 0.89 (t, J = 7 Hz, 3H).

The crude azide was reduced to the title amine as follows. To a magnetically stirred solution of 132 mg (0.43 mmol) of arachidonyl azide in 3 mL of dry diethyl ether was added 0.43 mL of a 1.0 M solution of lithium aluminum hydride (0.43 mmol) in THF dropwise at room temperature. The reaction mixture was refluxed for 3 h and then quenched with wet diethyl ether. The white suspension was filtered, and the filtrate was evaporated to dryness. Chromatography on silica gel (10–50% MeOH in dichloromethane) gave 65 mg (51%) of arachidonylamine as a colorless oil: TLC (EtOAc) R_f 1.50; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.38 (m, 8H), 2.82 (m, 6H), 2.70 (t, J = 6.6 Hz, 2H), 2.08 (m, 4H), 1.40 (m, 4H), 1.26 (m, 6H), 0.89 (t, J = 6.4 Hz, 3H). Anal. ($C_{20}H_{35}N$) C, H, N.

***N*-(3-Hydroxypropionyl)arachidonylamine (25).** To a magnetically stirred solution of 48 mg (0.17 mmol) of arachidonylamine in 2 mL of anhydrous dichloromethane was added 58 μL of a 2.0 M solution of trimethylaluminum (0.17 mmol) in hexane at room temperature. The mixture was stirred for 20 min, and then 12.24 mg (0.17 mmol) of β-propiolactone was added. The reaction mixture was refluxed for 6 h, quenched with 1 N HCl, and extracted with dichloromethane. The crude product was purified by column chromatography on silica gel (50–80% ethyl acetate in dichloromethane) to afford 51 mg (83%) of the title compound as an oil: TLC (EtOAc) R_f 0.26; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.35 (m, 8H), 3.85 (q, J = 5.4 Hz, 2H), 3.25 (q, J = 5.4 Hz, 2H), 2.84 (m, 6H), 2.66 (t, J = 6.8 Hz, 2H), 2.05 (m, 4H), 1.57 (m, 2H), 1.35 (m, 6H) 0.89 (t, J = 6.5 Hz, 3H). Anal. ($C_{23}H_{39}NO_2$) C, H, N.

***N*-(2-Acetoxyacetyl)arachidonylamine (26).** To a magnetically stirred solution of 75 mg (0.26 mmol) of arachidonylamine in 2 mL of dry dichloromethane was added 40 μL (0.37 mmol) of acetoxyacetyl chloride at room temperature, and the mixture was stirred for 1 h. Excess acetoxyacetyl chloride was destroyed by adding 50 μL of water, solvents were evaporated,

and the residue was chromatographed on silica gel (10–25% ethyl acetate in CH_2Cl_2) to afford 71 mg (70.2%) of the title amide as an oil: R_f (EtOAc) 0.79; ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.37 (m, 8H), 4.55 (s, 3H), 3.32 (q, J = 7 Hz, 2H), 2.81 (m, 6H), 2.18 (s, 3H), 2.09 (m, 4H), 1.50–1.25, (m, 6H), 0.92 (t, J = 7 Hz, 3H). Anal. ($C_{24}H_{39}NO_3$) C, H, N.

Radioligand Binding Assay. For CB1, rat forebrain membranes were prepared according to the procedure of Dodd et al.[37] The binding of the novel anandamide analogues to the cannabinoid receptor was assessed as previously described,[1,33] except that the membranes were treated with PMSF. Membranes, previously frozen at −80 °C, were thawed on ice. To the stirred suspension was added three volumes of 25 mM Tris-HCl buffer, 5 mM $MgCl_2$, and 1 mM EDTA, pH 7.4 (TME) containing 150 μM PMSF (made fresh in 2-propanol as a 100 mM stock). The suspension was incubated at 4 °C, and after 15 min a second addition of PMSF stock brought the concentration to 300 μM PMSF; then the mixture was incubated for another 15 min. At the end of the second 15-min incubation, the membranes were pelleted and washed three times with TME to remove unreacted PMSF. The treated membranes were subsequently used in the binding assay described below. Approximately 30 μg of PMSF-treated membranes were incubated in silanized 96-well microtiter plate with TME containing 0.1% essentially fatty acid-free bovine serum albumin (BSA), 0.8 nM [^{3}H]CP-55,940, and various concentrations of anandamide analogues in a final volume of 200 °C for 1 h. The samples were filtered using Packard Filtermate 196 and Whatman GF/C filterplates and washed with wash buffer (TME) containing 0.5% BSA. Radioactivity was detected using MicroScint 20 scintillation cocktail added directly to the dried filterplates, and the filterplates were counted using a Packard Instruments Top-Count. Nonspecific binding was assessed using 100 nM CP-55,940. Data collected from three independent experiments performed with duplicate determinations were normalized between 100% and 0% specific binding for [^{3}H]CP-55,940, determined using buffer and 100 nM CP-55,940. The normalized data was analyzed using a 4-parameter nonlinear logistic equation to yield IC_{50} values. Data from at least two independent experiments performed in duplicate were used to calculate IC_{50} values which were converted to K_i values using the assumptions of Cheng and Prusoff.[34]

For CB2 receptor binding studies, membranes were prepared from frozen mouse spleen essentially according to the procedure of Dodd et al.[37] Silanized centrifuge tubes were used throughout to minimize receptor loss due to adsorption. The CB2 binding assay was conducted in the same manner as for CB1.

[^{35}S]GTPγS Binding Assay. 1. Cerebellar Membrane Preparation. The procedure was adapted from the method of Dodd et al.[37] The stripped rat brains were slightly thawed, and using a spatula, the cerebellum was removed and discarded; the remaining tissue was homogenized in ice-cold homogenization buffer (0.32 M sucrose, 10 mM Tris, 5 mM EDTA, pH 7.4). The homogenate suspension was centrifuged at 3700g for 10 min. The supernatant was decanted, and 12 mL was layered over 10 mL of 1.2 M sucrose. These tubes were centrifuged in a L7-65 ultracentrifuge using a 50.2 Ti rotor at 4 °C for 29 min at 44 000 rpm. The layer at the interface was then removed and subjected to a second sucrose spin over 0.8 M sucrose. The pellet was resuspended in TME buffer (25 mM Tris base, 5 mM $MgCl_2$, 1 mM EDTA, pH 7.4), aliquoted, and stored at −70 °C. Protein was determined using the method of Markwell et al.[40]

2. [^{35}S]GTPγS Binding Assay. Our assay was based on a method by Selley et al.[38] and was adjusted for a 96-well plate analysis. Briefly, the rat membrane preparation (40–50 μg of protein) was incubated for 1 h at 30 °C in assay buffer (10 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, 0.1% BSA) with 50 μL of 50 μM GDP, 50 μL of 0.05 nM [^{35}S]GTPγS, or 100 μL of either; 10 μM GTPγS was used to measure the nonspecific binding, a series of different concentrations of the analogues being tested, or buffer alone as a control to obtain the baseline of GTPγS stimulation. The reaction was terminated by rapid filtration through Whatman GF/B filters, with ice-cold wash buffer containing 0.5% bovine serum albumin using the Packard Filtermate. Bound radioactivity was measured on the Packard Top-Count microplate scintillation counter.

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